# Increased Susceptibility to Mycoplasma Infection of SV40 Transformed Human Amnion Cells[1] (35608)

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Various effects of mycoplasma infection on SV40 transformed human amnion cells have been reported (1–4). Reduced incidence of transformation, delayed appearance of transformed foci, a reduction in the number of population doublings and of the time of cultivation prior to "crisis" was, in part, interpreted as results of the cell destructive effects of mycoplasma. SV40 transformed amnion cells, when mycoplasma-infected in serial subculture, showed more cell destruction than mycoplasma and SV40 infected primary cultures, and the effects on population doublings were more pronounced (2). Mycoplasma elimination reduced the cell destructive effects. After concurrent mycoplasma–SV40 infection, chromosome numbers were maintained in the diploid range for a longer period than in the absence of mycoplasma, and most abnormalities were present at lower frequencies (4). These changes were not reversed after mycoplasma elimination. Since primary amnion cells are highly resistant to infection by mycoplasma (5) and spontaneously transformed FL human amnion cells are highly susceptible (5, 6), it was important to study the susceptibility of SV40 transformed human amnion cells by similar criteria. In the present investigation, the response to mycoplasma infection of pre- and post-"crisis" amnion cells, transformed by SV40, were compared, by growth curve studies and morphological observations, with the response of primary amnion cells from which they were derived.

*Materials and Methods. Virus transformed cells.* The SV40 strain VA 45-54 GMK 4 used for infection of primary amnion cultures has been previously described (7). Many of the characteristics of pre-"crisis" strains (7–10) and post-"crisis" lines (11) of SV40 transformed amnion cells and conditions for their cultivation have also been reported.

*Mycoplasma.* The mycoplasma HT strain, its origin (6), identification (2), and methods for detection (6, 12), have been reported. Quantitative determinations of cell-associated mycoplasma were based on direct microscopic examination with phase optics of cell cultures exposed to hypotonic treatment, fixation, and orcein staining (12, 13). Procedures for determining the concentration of colony-forming units (CFU), have been described (2).

*Growth curve studies.* Tubes were inoculated with a constant number of cells, removed from stock cultures by trypsinization. The number of cells per tube culture, containing 2 ml of medium was determined at regular intervals on trypsinized cell suspensions, using a hemacytometer for cell counts.

*Effects on cell morphology.* Cell morphology was examined on cultures after fixation with Bouin's solution for 15 min and staining with hematoxylin and eosin.

*Results.* Mycoplasma effects on the growth of SV40 transformed human amnion cells were observed by comparing the appearance and structure of the cell colonies in infected and uninfected cultures, seeded with 2000 cells (Fig. 1). In uninfected cultures, the colony centers were built up in several to many layers by a pronounced vertical growth tendency. Mycoplasma infection, 4 days after cell seeding, not only reduced the number of cells per colony, but, with a few exceptions, the cells grew as a monolayer within the colonies. This change was apparent for

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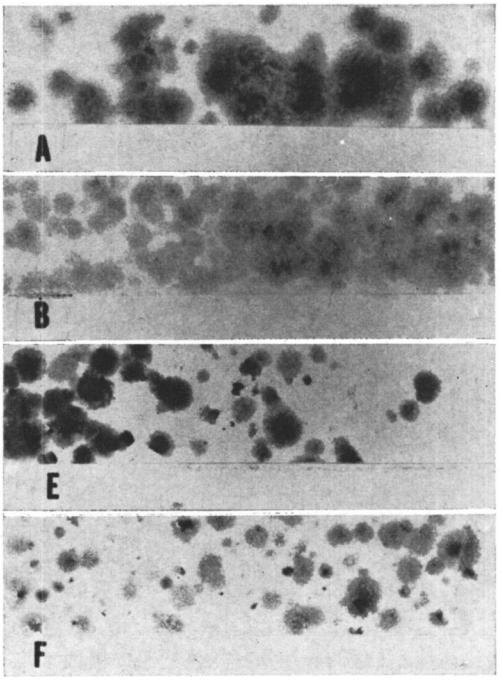



FIG. 1. Colonies of SV40 transformed human cells, cultured on coverslips in Yerganian tubes; hematoxylin and eosin; 0.875×; (A) pre-"crisis" cells (A261) passage 12 after SV40 infection, 25-day culture; (B) comparable culture, infected with mycoplasma for 21 days; (E) post-"crisis" cells (A244), passage 64 after SV40 infection (passage 44 after "crisis"), 25-day culture; (F) comparable culture, infected with mycoplasma for 21 days.

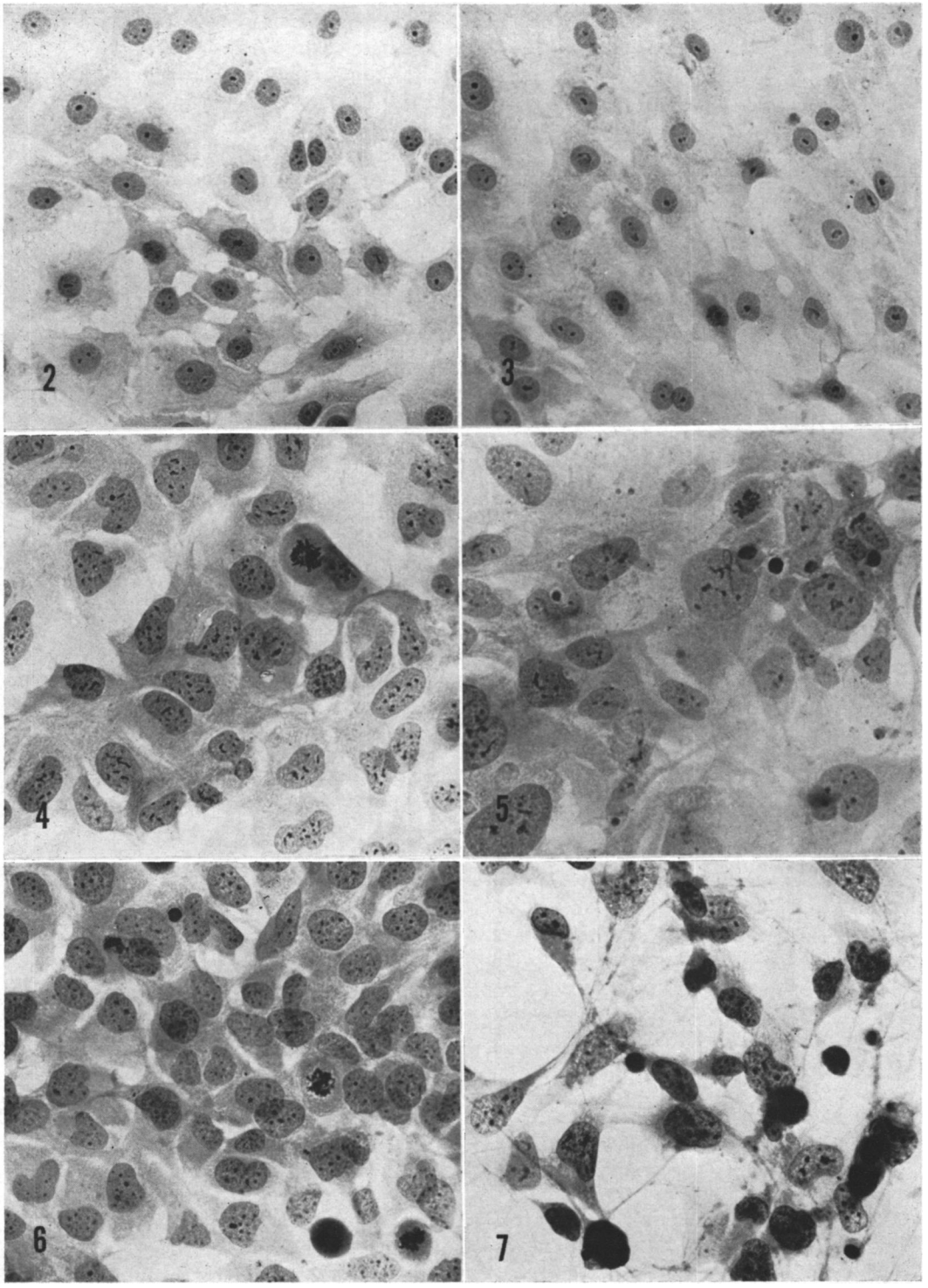
pre-"crisis" cells, as well as for cells of an established line, recovered from "crisis." The reduction of growth per colony was more pronounced in the infected cultures of post-"crisis" cells. Both types of cells had a low cloning efficiency which was not significantly affected by the mycoplasma infection under these conditions.

When similar numbers of pre- or post-"crisis" transformed cells were inoculated into cultures of primary amnion cells, the cloning efficiency increased significantly (to approx 10%). The colony numbers were not affected by mycoplasma infection (at day 4 after cell seeding) during the first 2 weeks. However, 3 weeks after infection, the cultures inoculated with the post-"crisis" cells, macroscopically appeared void of colonies. At this time, colonies of pre-"crisis" cells were still clearly visible, although greatly reduced in size, and their numbers were similar to those in the uninfected control cultures.

From microscopic examination of fixed and stained cultures, it was apparent that the reduced amounts of growth in the mycoplasma-infected cultures of transformed cells were correlated with cytopathic cell changes and cell destruction. The pre-"crisis" cells (Fig. 4) grew less organized after infection (Fig. 5) and showed nuclear changes, including enlargement, irregular shapes, and pyknosis. Phagocytosis of dead cell material was prevalent. Post-"crisis" cell cultures (Fig. 6) showed much more pronounced destruction after infection (Fig. 7). The total appearance of the cell layer was changed from an epithelial-like growth to a completely unorganized culture of spindle-shaped cells containing oblong nuclei of irregular shapes, mixed with dead cells and debris in a network of thin intercellular connections. In contrast, the morphology of primary amnion cells (Fig. 2) under similar culture conditions and mycoplasma infection (Fig. 3), appeared completely unchanged.

Obvious effects were also seen by microscopy of the mycoplasma-infected mixed cultures. Transformed pre-"crisis" cells in uninfected cultures were of epithelial-like morphology and grew at a high density in several layers. The border line between the colony and the surrounding primary amnion cells was not distinct, as observed for colonies of FL cells in a primary amnion cell culture (5). Colonies of post-"crisis" cells showed a very similar picture in uninfected cultures. In mycoplasma-infected cultures of pre-"crisis" and primary amnion cells, observed 3 weeks after infection, the enlarged transformed cells in the colonies appeared healthy, although mitoses were infrequent. In contrast, the very few remaining cells in the colonies in the mixed cultures of post-"crisis" cells and primary amnion cells, infected for the same period, were highly irregular in shape, demonstrated cytopathic effects and appeared to be overgrown by the primary cells, which in all the infected cultures looked unaffected.

Similar experiments carried out with cells of several pre-"crisis" strains and different post-"crisis" lines have confirmed these effects.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FIGS. 2–7. Hematoxylin and eosin stained cultures; 321×: (2,4,6) uninfected; (3,5,7) infected with mycoplasma for 21 days; (2 and 3) 36-day-old primary amnion cell cultures; (4 and 5) SV40 transformed amnion cells, pre-"crisis", passage 12 after SV40 infection; and (6 and 7) SV40 transformed amnion cells, post-"crisis", passage 64 after SV40 infection (passage 44 after "crisis").

Several growth curve experiments confirmed the cell destructive effects of the mycoplasma infection and supported the morphological observation that post-"crisis" cells were more susceptible to mycoplasma infection than pre-"crisis" cells. Two experiments (Expts. 1 and 2) are shown in Fig. 8. The uninfected early pre-"crisis" passage of strain A274 in Expt. 1 reached a maximal cell density, 600,000 cells/culture, at day 10 after seeding. The uninfected late pre-"crisis" passage of strain A265 in Expt. 2 continued to increase in cell density for several more days, as did the uninfected post-"crisis" lines in both experiments, to a density of approximately $2 \times 10^6$ cells/culture. After high dose mycoplasma exposure in Expt. 1, effects on cell numbers were notable already at day 6 after infection; both pre- and post-"crisis" cell numbers were reduced, post-"crisis" more than pre-"crisis." This pattern became more pronounced in the following days. The highest reduction of cell number was observed at day 15, at which the post-"crisis" line contained nearly 30 times fewer cells than its uninfected control. After the lower mycoplasma dose exposure in Expt. 2, cell numbers were not reduced until day 10 after infection. From day 13, the difference was more pronounced for the post-"crisis" line. Maximum reduction for the post-"crisis" line was 20-fold.

The number of colony-forming units of mycoplasma was determined on supernatants from cultures of pre-"crisis" strain A274, passage 10, post-"crisis" line H(as), passage 69 after "crisis", and primary amnion cells. All cultures were grown in T-9 flasks in McCoy's medium 5a containing 10% agamma calf serum or in LY medium (14) with 20% human serum. Fresh, cell-free media of the two types served as controls. Two days after cell seeding the cultures were infected with mycoplasma, each culture receiving $4.2 \times 10^6$ CFU. Half of the 2 ml of culture fluid in all the cultures was removed at days 3, 5, 7, 10, and 12 after infection, and samples of culture fluid were collected for determination of

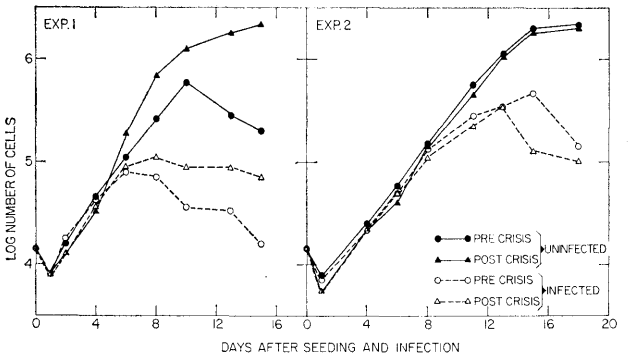



FIG. 8. Number of uninfected and mycoplasma-infected pre-"crisis" and post-"crisis" SV40 transformed amnion cells as a function of time after seeding and infection. In McCoy's medium 5a (19) containing 10% agamma calf serum, 15,000 cells were seeded/tube. Half of the cultures were inoculated with mycoplasma, $7.2 \times 10^5$ CFU/tube in Expt. 1; $1.4 \times 10^3$ CFU in Expt. 2. Culture medium was replaced every other day. Expt. 1 compares an early passage (passage 9) of pre-"crisis" strain A274, with post-"crisis" line E $Mel_3$, passage 60 after "crisis". Expt. 2 compares a late passage (passage 20) of pre-"crisis" strain A265 with post-"crisis" line A244, passage 71 after "crisis."

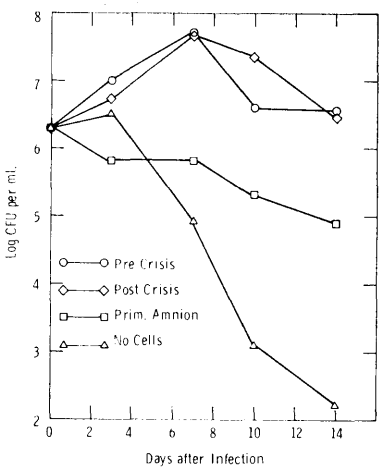


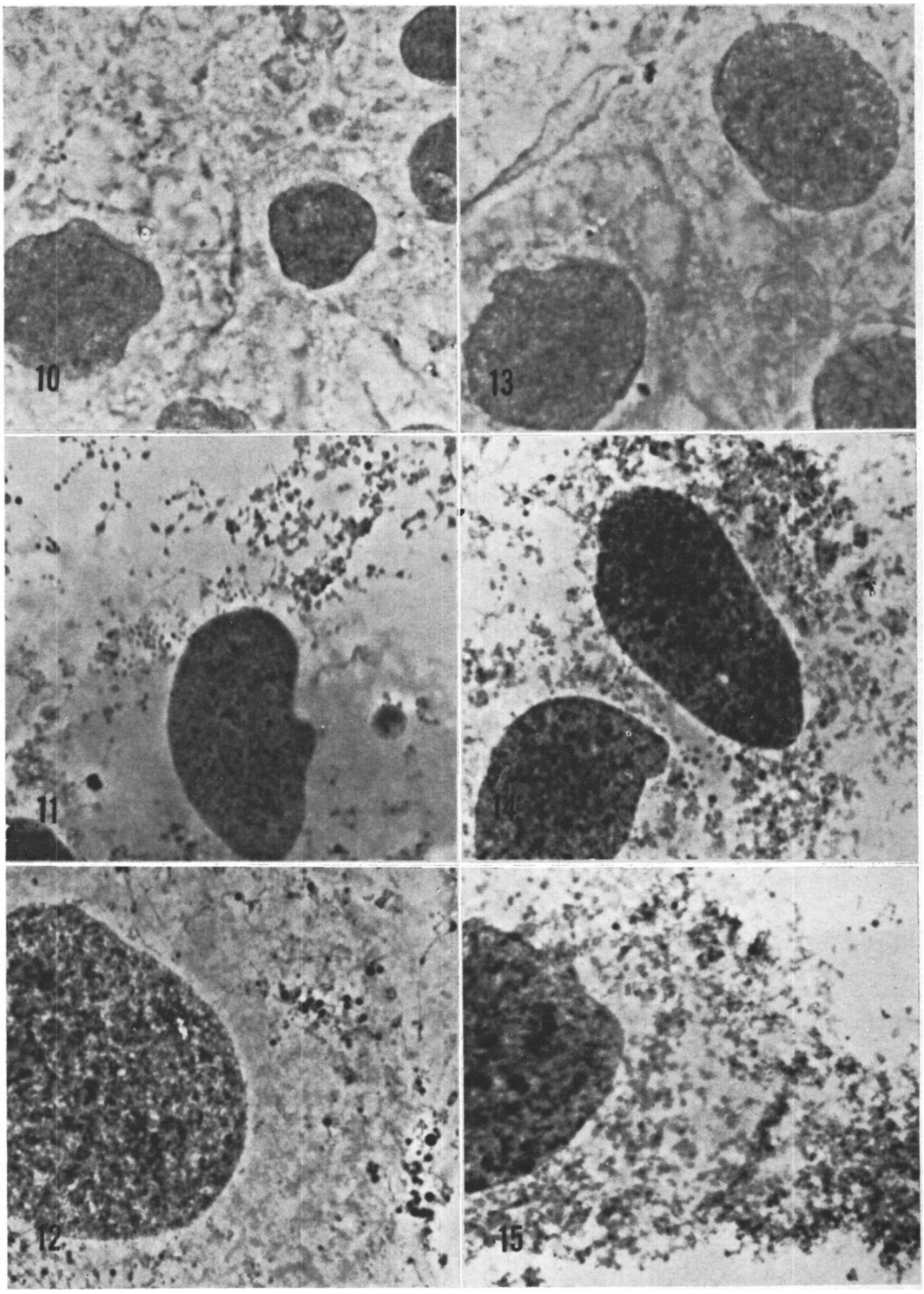

FIG. 9. Colony-forming units of mycoplasma per milliliter in the culture supernatants of pre-"crisis" and post-"crisis" SV40 transformed amnion cells, primary amnion cells, and in the medium (McCoy's with 10% agamma calf serum) as a function of time after infection.

CFU per ml at days 3, 7, 10, and 14.

The results for the cultures in McCoy's medium with agamma calf serum are shown in Fig. 9. During the first week, CFU per ml increased from $2.6 \times 10^6$ to $5.0 \times 10^7$ for the pre-"crisis" cell cultures. During the next week the titers decreased to the level of the inoculum. All titers for the post-"crisis" cultures were in the same range. For primary amnion cultures the titers decreased during the experimental period and were from 1 to 2 logs lower than for the transformed cell cultures. Titers decreased rapidly after the third day when cells were absent; at day 14 they were more than 4 logs lower than those for the transformed cell cultures. It might be important that the titers at day 3 for the primary amnion cultures were approximately 0.5 log lower than those in the absence of cells. In sharp contrast to these results, CFU per milliliter for the cultures of all three cell types and for those without cells were within the same range ($1.2 \times 10^6$ to $2.9 \times 10^7$ CFU/ml) when LY medium with 20% human serum was used. Except for the day 14 point at which the titer in the absence of cells represented the lowest titer (still within the indicated range) there was no indication from the data that the presence of cells in this medium increased the amount of free mycoplasma. Consequently, the data in Fig. 9 show that the presence of primary amnion cells support the propagation of mycoplasma to a much lesser extent than any of the SV40 transformed amnion cell types.

The amounts of cell-associated mycoplasma for the three cell types in either of the two medium–serum combinations, 6 days after mycoplasma infection with similar inocula, are illustrated in Figs. 10 to 15. In McCoy's medium with agamma calf serum, very few cell-associated mycoplasma were observed on primary amnion cells (Fig. 10), whereas pre-"crisis" cells (Fig. 11) and post-"crisis" cells (Fig. 12) showed sizable amounts of mycoplasma. In LY medium with 20% human serum, primary amnion cells (Fig. 13) also showed very limited amounts of cell-associated mycoplasma. In this medium, pre-"crisis" cells (Fig. 14) and post-"crisis" cells (Fig. 15) showed a heavy coverage with cell-associated mycoplasma. The amount of cell-associated mycoplasma appeared similar for the pre-"crisis" and post-"crisis" cells when they were cultured in the same medium.

*Discussion.* Previous studies (5) have shown that morphological changes were inconspicuous and cell density was not affected during many weeks after infection of primary amnion cells with the HT strain of mycoplasma fermentans. The cytopathogenicity of this strain for amnion cells transformed by SV40 has also been reported (2, 3). SV40 transformed cells which had been infected with mycoplasma in primary culture maintained a relative resistance to mycoplasma during serial cultivation, as compared to transformed cells infected with mycoplasma after a number of subcultures. The differences in degrees of cell destruction between primary and transformed cells was interpreted as results of a cell change associated with the SV40 transformation. There were reasons to explain the relative resistance maintained after an early mycoplasma infection as the result of mycoplasma-induced genetic changes. The present data have added to this picture that SV40 transformed amnion cells recovered from "crisis" are highly susceptible to mycoplasma infection, even




FIGS. 10–15. Cultures infected with mycoplasma for 6 days. Exposed to hypotonic treatment, Carnoy's fixative, and orcein staining (12); phase optics; 1,280×: (10,11,12) in McCoy's medium with 10% agamma calf serum; (13,14,15) in LY medium with 20% human serum; (10 and 13) primary amnion cells; (11 and 14) SV40 transformed amnion, pre-"crisis", passage 12 after infection; and (12 and 15) SV40 transformed amnion cells, post-"crisis."

more than pre-"crisis" strains infected during subcultivation. The high susceptibility is expressed by the increase in degree of cytopathic effect and in the substantial cell losses encountered.

The high resistance to mycoplasma infection of primary amnion cells is correlated with a very low amount of cell-associated mycoplasma and with relatively low titers of free mycoplasma in McCoy's medium with agamma calf serum which does not support the mycoplasma propagation well in the absence of cells. In contrast, substantial amounts of cell-associated mycoplasma are seen on both the pre- and post-"crisis" cell types in this medium. The amounts increase in LY medium with human serum, which also supports the propagation of free mycoplasma well, but primary amnion cells are still mainly void of mycoplasma. Titers of free mycoplasma in McCoy's medium in the absence of cells are actually higher than those in the presence of primary amnion cells at day 3 after infection; however, later during the experiment they decrease rapidly. These different situations can be interpreted as follows:

Compared to McCoy's medium with agamma calf serum, certain components, including yeast extract (15), contained in LY medium with human serum are highly favorable for the mycoplasma propagation in the absence of cells, and titers are not increased when any of the three types of cells are present in the LY medium. It has been shown for other systems that the presence of cells increase the amount of mycoplasma in the culture supernatant, either by providing certain nutrients, by inactivating substances that prevent growth, or by conditioning the medium and assisting in proper pH adjustment (16). This function of the pre- and post-"crisis" cells can be observed in the less optimal McCoy's medium with agamma calf serum. The presence of primary amnion cells may provide similar advantages for mycoplasma propagation, although this is not as apparent.

It has been shown previously (16) that mycoplasma selectively favor the cell site, rather than the fluid phase, for their location of propagation, and that a considerable proportion of the mycoplasma present in the culture fluid is derived from cell-associated replication. Apparently there are more favorable conditions (nutritional a.o.) in association with the mammalian cell. A major reason for the low numbers of colony-forming units observed in the culture fluid of primary amnion cultures may therefore be related to their conspicuously low amount of cell-associated mycoplasma which again could reflect a different cell surface structure, for example, lack of receptors (16). The difference in titers at day 3 between culture fluid from amnion cells and medium without cells, however, indicate that presence of amnion cells or substances they produce might actually inhibit mycoplasma propagation.

The similarity in the amounts of cell-associated and free mycoplasma in cultures of pre- and post-"crisis" transformed cells under different medium and serum conditions shows that their differences in susceptibility to mycoplasma infection is not related to different amounts of mycoplasma present. As previously pointed out (5), the considerable resistance of primary amnion cells might well be the result of the pronounced absence of cell-associated mycoplasma. Even when substantial amounts of free mycoplasma are present in the LY medium and nutritional deficiencies (15) occur, amnion cells are not destroyed.

Since there are reasons (8) to characterize the SV40 transformed pre-"crisis" cells as premalignant cells which after "crisis" survival are comparable to the cells in the developed malignant tumor (17), the difference in susceptibility to mycoplasma infection among these three cell types may be an expression of the different state of *in vitro* malignancy. Spontaneously transformed amnion cells (FL a.o.) with pronounced malignant characteristics are also highly susceptible to mycoplasma as are cells of many continuous lines (6). It is generally known that primary cultures of normal cells, also of other cell types, rarely become contaminated with mycoplasma (18). It has been assumed that the short exposure to laboratory handling as compared to the repeated exposure of continued cell lines might explain this difference. In view of the present data, however, it is conceivable that the normal cell is genuinely

resistant and does not support the propagation of mycoplasma to the extent of cells which have changed towards malignancy.

The difference in susceptibility to mycoplasma infection between pre- and post-"crisis" lines may be of practical importance in future experiments on the phenomenon of "crisis" and post-"crisis" establishment. This difference may serve as a basis for methods by which cells of these two stages of transformation can be distinguished.

*Summary.* The effects of infection with mycoplasma (strain HT, mycoplasma fermentans) on cell morphology and growth were different for primary amnion cells and for SV40 transformed amnion cells before and after "crisis." Post-"crisis" cells showed distinctly more growth reduction, cytopathic changes, and cell destruction than pre-"crisis" cells, although cell-associated mycoplasma was present in similar, high amounts. Pronounced resistance of primary amnion cells was correlated with a very low amount of cell-associated mycoplasma, even at high concentrations of free mycoplasma. The presence of the primary cells supported mycoplasma propagation, as determined in the culture fluid, much less than either pre- or post-"crisis" cells. There is indication that the primary cells may be responsible for a mechanism of inhibition of mycoplasma propagation. It is suggested that the differences in mycoplasma susceptibility among the three cell types are related to their different state of *in vitro* malignancy, and may serve as useful criteria in further studies of the changes occurring after the virus transformation.

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